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Synergistic catalysis in an artificial enzyme by simultaneous action of two abiological catalytic sites

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Abstract

Artificial enzymes, which are hybrids of proteins with abiological catalytic groups, have emerged as a powerful approach towards the creation of enzymes for new-to-nature reactions. Typically, only a single abiological catalytic moiety is incorporated. Here, we introduce a design of an artificial enzyme that comprises two different abiological catalytic moieties and show that these can act synergistically to achieve high activity and enantioselectivity, up to >99% e.e., in the catalyzed Michael addition reaction. The design is based on the transcriptional regulator LmrR as the protein scaffold and combines a genetically encoded unnatural p-aminophenylalanine residue, which activates an enal through iminium ion formation, and a supramolecularly bound Lewis acidic Cu(II) complex, which activates the Michael donor by enolization and delivers it to one preferred prochiral face of the activated enal. This study demonstrates that synergistic combination of abiological catalytic groups is a powerful approach to achieving catalysis normally outside the realm of artificial enzymes.

Main Text

The drive for a more sustainable approach to chemical synthesis has spurred the development of artificial enzymes for reactions that have no equivalent in nature.[1,2] A key challenge in the design of such artificial enzymes is the creation of the active site. An important approach involves the introduction of abiological catalytically active moieties, which could be transition metal complexes or organocatalytic groups,[3–7] in stable protein scaffolds that give rise to basal level of activities, that can then be improved by fine-tuning the protein environment by mutagenesis.[8,9] Current efforts towards the creation of artificial enzymes have focused exclusively on introducing a single abiological catalytic moiety.[3] However, the enviable rate accelerations and selectivities achieved by natural enzymes in part relate to nature's ability to combine multiple catalytic strategies in a synergistic fashion.[10] Here, we now report an artificial enzyme containing two abiological catalytic moieties that engage in synergistic catalysis to achieve highly enantioselective Michael addition reactions.

Synergistic catalysis is a concept in which two substrates of a bimolecular reaction, e.g. an electrophile and nucleophile, are activated simultaneously by separate catalytic moieties. This causes a dramatic lowering of the HOMO−LUMO gap, which translates into a significant acceleration of the reaction.[11,12] It is increasingly applied in homogeneous catalysis, for example in the combination of transition-metal catalysis and amino organocatalysis.[13–16] Yet, the catalytic efficiency of such approaches is inherently limited by the entropic cost of bringing two activated substrates together in a productive orientation.[17] In contrast, placement and orientation of reactive groups in the defined space of an active site is a hallmark of enzymes. Hence, we envisioned that placing two abiological groups at judicious positions in a stable protein scaffold would result in a highly efficient and selective artificial enzyme.

Our design of the artificial enzymes is based on the Lactococal multidrug resistance Regulator (LmrR), a small homodimeric transcription regulator from *L. lactis* that has been demonstrated to be a remarkably versatile scaffold for the design and creation of artificial (metallo-)enzymes (Figure 1-a).[18–20] The choice for this protein relates to its attractive structure: it contains an unusually large hydrophobic pocket at the dimeric interface with at its center two tryptophan moieties W96 and W96', one from each monomer, that are key contributors to its binding of guest molecules via π stacking interactions. We have previously taken advantage of this promiscuous guest binding for introduction of abiological metal cofactors such as Cu(1,10-phenanthroline)$(NO_3)_2$ (Cu(II)-phen) by supramolecular interactions. This resulted in an artificial metalloenzyme for enantioselective conjugate addition of indoles to enones, in which the Cu(II)-phen cofactor acts as a Lewis acid for the activation of the enone (Figure 1-b).[21]

An alternative approach involves introduction of catalytic groups by using expanded genetic code methods, i.e. stop codon suppression.[22–24] Recently we have reported on the application of this methodology for the creation of a designer enzyme featuring a unnatural catalytic p-aminophenylalanine (pAF) residue,[4,5] Here, the aniline side chain of pAF was used as nucleophilic catalyst for formation of hydrazones from aldehydes (Figure 1-c). The reaction involves the transient formation of an iminium ion intermediate, which is a common activation strategy in many organocatalytic reactions.[25] The central tryptophan residues in this case are proposed to contribute to substrate binding. We envisioned that both these design approaches can be combined into a single protein, to create an artificial enzyme with two abiological catalytic sites capable of acting simultaneously.

Here we report a design of such an artificial enzyme comprising two different abiological catalytic moieties and show that these can act synergistically for the catalysis of enantioselective Michael addition reactions. In our design an α,β-unsaturated aldehyde is activated through iminium

ion formation with the aniline residue, while the enolate is formed by activation of a ketone precursor by the Lewis acidic Cu(II)-phenanthroline complex to give the Michael addition product (Figure 1-d). The nucleophile bound copper complex will be bound between the tryptophan moieties, resulting in delivery of the enolate to one preferred prochiral face of the activated enone. These artificial enzymes give rise to high activity and enantioselectivity, up to >99% e.e, in the catalyzed Michael additions reactions.

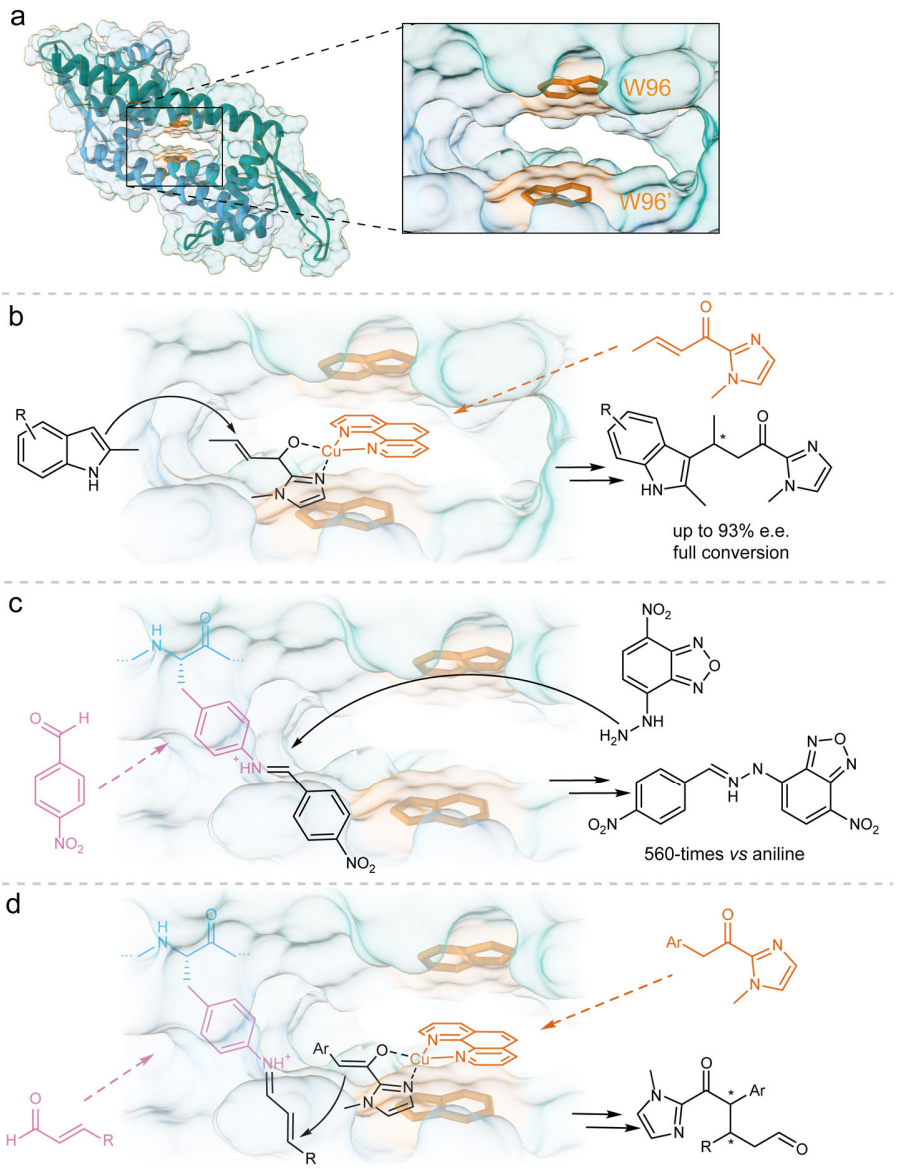


Figure 1. Artificial enzyme designs based on LmrR. (a) The promiscuous binding pocket of LmrR with one pair of central tryptophan residues. (b) Previous work based on LmrR: Enantioselective Friedel-Crafts reaction catalyzed by Artificial metalloenzymes which is recruiting Cu(II)-phenanthroline to the LmrR pore by supramolecular assembly. (c) Designer enzyme with an unnatural catalytic aniline residue for hydrazone formation. (d) This work: Asymmetric Michael addition reaction catalyzed by synergistic combination of two catalytic sites: a pAF residue for the activation of the enal and Cu(II)-phenanthroline for the generation and delivery of the enolate nucleoephile.

Results

Creation of the artificial enzyme. The LmrR variant used in this study, referred to as LmrR_V15pAF, contains a non-canonical pAF residue at position 15 inside the hydrophobic pore.[4] The pAF residue can be introduced directly using the dedicated orthogonal translation system,[26] but in our experience it is most practical to use the system for incorporation of p-azidophenylalanine (pAzF), followed by Staudinger reduction with tris(2-carboxyethyl)phosphine (TCEP), which was added to the protein solution after STREP-tag affinity purification, but before the final dialysis step. Hence, no additional handling steps were required compared to direct incorporation of pAF.

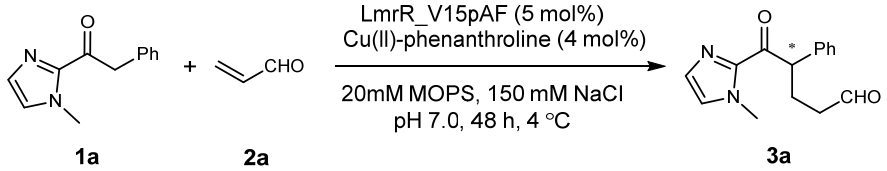
The Lewis acidic site was then introduced through supramolecular self-assembly, by combining LmrR_V15pAF with Cu(II)-phen in a buffered solution (20 mM MOPS, 150 mM NaCl, pH 7.0). The binding affinity of Cu(II)-phen to LmrR_V15pAF was determined by titration through monitoring of the quenching of tryptophan fluorescence. A dissociation constant (K_d) of 0.53 μM was determined for binding of Cu(II)-phen to LmrR_V15pAF (Supplementary Figure 4), which is similar to the K_d reported for the binding of Cu(II)-phenanthroline to LmrR. This means the pAF residue does not negatively affect the binding of Cu(II)-complex to the two tryptophan residues (W96/W96').

Catalysis studies. The catalytic potential of the new artificial enzyme was evaluated in the enantioselective Michael addition reaction. While examples of promiscuous and designed enzyme employing iminium ion activation strategies for the catalysis of Michael additions have been reported, these generally involve the use of readily enolizable Michael donors for which no additional activation step is required.[6,27–29] Here, we used the much less reactive ketone 1-(1-

methyl-1H-imidazol-2-yl)-2-phenylethan-1-one (**1a**) as Michael donor. This ketone does not enolize spontaneously under the reaction conditions, but this can be achieved by binding to a Lewis acidic metal complex.[30,31] Addition to acrolein (**2a)** then gives the Michael addition product **3a**.[32]

Less than 10% e.e. and a poor yield were obtained when using MacMillan-type chiral secondary amine catalysts for this reaction (Supplementary Figure 2). With other common amine organocatalyst(s) also a poor yield of product was obtained, while using these organocatalysts in combination with Cu(II)-phen, in absence of protein, did not result in product formation (Figures S1, S2). These results suggest most amine catalysts are incompatible with the Cu(II)-phen complex. In contrast, using LmrR_V15pAF/Cu(II)-phen, prepared by self-assembly from 4 mol% of Cu(II)-phen (40 μM) with a slight excess (1.25 equiv) of LmrR_V15pAF (50 μM), the Michael addition product **3a** was obtained in 36% yield with 86% e.e. (Table 1, entry 5). That the combination of the two catalytic sites is required for this reaction is evident from the fact that no product was formed when omitting one of the components from the catalyst, that is, using only LmrR_V15pAF or Cu(II)-phen or the combination of wild type LmrR, without pAF residue, and Cu(II)-phen (Table 1, entry 1–3). Using LmrR_V15pAF with $Cu(NO_3)_2$ as catalysts in the reaction resulted in 10% e.e. of **3a** (Table 1, entry 4). This supports our hypothesis that the binding of copper complex to the two central tryptophan residues, which is mediated through the phenanthroline ligand,[21] is important for the reaction to occur enantioselectively. Using copper complexes of ligands other than phenanthroline gave rise to lower enantioselectivities in the catalyzed reaction, as did the use of other metal phenanthroline complexes (Supplementary Table 1).

Table 1. Results of Michael addition reactions catalyzed by LmrR_V15pAF/Cu(II)-phen



Entry	Catalysts		Yield (%)[a]	e.e. (%)[b]
1	LmrR_V15pAF	/	<1	ND
2	/	Cu(II)-phen	<1	ND
3	LmrR	Cu(II)-phen	<1	ND
4	LmrR_V15pAF	$Cu(NO_3)_2$	25 ± 8	10 ± 2
5	LmrR_V15pAF	Cu(II)-phen	36 ± 2	86 ± 1
6[c]	LmrR_V15pAF	Cu(II)-phen	27 ± 3	81 ± 1

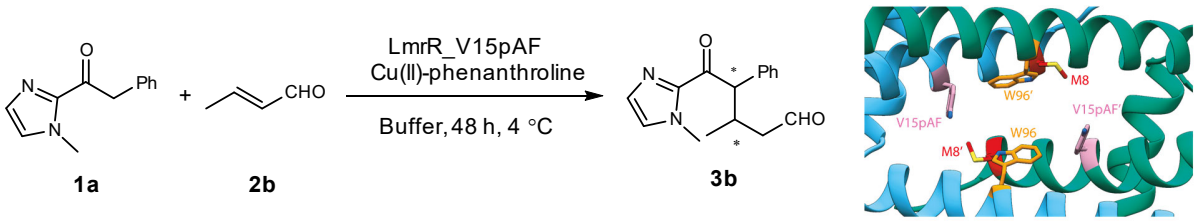
Typical conditions: 0.8 equiv Cu(II)-phen (4 mol%; 40 μM) loading with respect to LmrR_V15pAF or LmrR (5 mol%; 50 μM), 1 mM **1a**, 10 mM **2a**, in 20 mM MOPS buffer (pH 7.0), 150 mM NaCl, at 4 °C for 48 h, unless noted otherwise. Yield and e.e. values are the average of at least two independent experiments, both carried out in duplicate. All error values are given as standard deviation. [a]Yields are determined by HPLC analysis. [b]e.e. values are determined by chiral HPLC. [c]Reaction with 0.8 equiv Cu(II)-phen (2 mol%; 20 μM) loading with respect to LmrR_V15pAF (2.5 mol%; 25 μM).

Encouraged by these results, we performed this reaction with crotonaldehyde instead of acrolein as Michael acceptor, which results in the simultaneous formation of two chiral centers. To our delight, the reaction with crotonaldehyde gave excellent enantioselectivities for both diastereomers with 98% and 86% e.e., respectively, when using the combination of LmrR_V15pAF with Cu(II)-phen (Table 2, entry 4). Using LmrR in combination with Cu(II)-phen and aniline, or another external chiral secondary amine catalyst (Table 2, entry 2 and Supplementary Figure 3,) gave no reaction, while using $Cu(NO_3)_2$, instead of Cu(II)-phen, in combination with LmrR_V15pAF resulted in dramatically lower e.e., d.r. and yield (Table 2, entry 3). These results further support that physical separation of the aniline and Cu(II) complex, as well as precise positioning of these two with respect to each other, are important to achieve enantioselective catalysis.

Further optimization of the reaction conditions gave rise to an increased yield and similar enantioselectivity (Supplementary Table 2) when using a small excess of Cu(II)-phen with respect

to LmrR_V15pAF at pH 6 (Table 2, entry 4). The kinetics of the reaction was measured as a function of crotonaldehyde concentration (1-25 mM), at a fixed ketone **1a** concentration (1 mM). Saturation kinetics was observed and the initial rates were fitted to the Michaelis Menten equation, giving an apparent catalytic efficiency ($k_{cat}/K_{M(crotonaldehyde)}$) of 0.115 ± 0.017 M^{-1} s^{-1}, an apparant k_{cat} of (2.58 ± 0.38) x 10^{-3} $s^{-1}$and a $K_{M(crotonaldehyde)}$ of 22.5±5.84 mM (Supplementary Figure 6). The latter value is comparable to the K_M measured for benzaldehyde derivatives in the recently reported hydrazone formation reaction catalyzed by LmrR_V15pAF, suggesting similar binding interactions play a role.[5]

Table 2. Optimization of reaction conditions and mutagenesis study for artificial enzymes



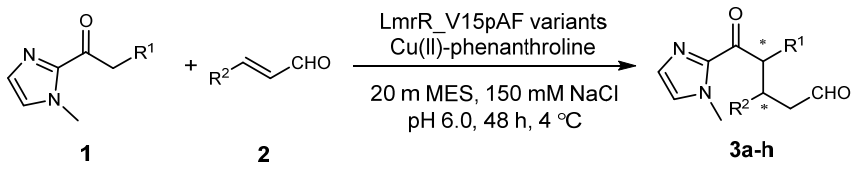
Entry	Catalysts		Yield (%)[a]	d.r. [b]	e.e. (%)[b]
1	LmrR	Cu(II)-phen	<1	ND	ND
2[c]	LmrR + aniline	Cu(II)-phen	<1	ND	ND
3	LmrR_V15pAF	$Cu(NO_3)_2$	14 ± 3	1 : 1	57 ± 2/39 ± 1
4	LmrR_V15pAF	Cu(II)-phen	54 ± 2	3.5 : 1	98 ± 0/86 ± 2
5[c]	LmrR_V15pAF	Cu(II)-phen	65 ± 1	4 : 1	98 ± 0/86 ± 1
6[d]	LmrR_V15pAF	Cu(II)-phen	40 ± 2	4 : 1	98 ± 0/84 ± 1
7[e]	LmrR_V15pAF	Cu(II)-phen	15 ± 1	4 : 1	98 ± 0/84 ± 0
8[f]	LmrR_V15pAF	Cu(II)-phen	6 ± 1	4 : 1	99 ± 0/77 ± 1
9[c]	LmrR_V15pAF_W96A	Cu(II)-phen	12 ± 2	1.2 : 1	82 ± 1/49 ± 2
10[c]	LmrR_V15pAF_M8R	Cu(II)-phen	34 ± 3	4 : 1	98 ± 0/81 ± 1
11[c]	LmrR_V15pAF_M8D	Cu(II)-phen	61 ± 1	3 : 1	96 ± 0/62 ± 2
12[c]	LmrR_V15pAF_M8W	Cu(II)-phen	64 ± 3	7 : 1	99 ± 0/84 ± 2
13[c]	LmrR_V15pAF_M8I	Cu(II)-phen	76 ± 2	6 : 1	>99 ± 0/88 ± 1
14[c]	LmrR_V15pAF_M8L	Cu(II)-phen	82 ± 1	6 : 1	>99 ± 0/93 ± 1
15[d]	LmrR_V15pAF_M8L	Cu(II)-phen	68 ± 2	6 : 1	>99 ± 0/93 ± 1
16[e]	LmrR_V15pAF_M8L	Cu(II)-phen	36 ± 1	5.4 : 1	>99 ± 0/92 ± 0
17[f]	LmrR_V15pAF_M8L	Cu(II)-phen	15 ± 2	5 : 1	>99 ± 0/90 ± 0

Typical conditions: 0.8 equiv Cu(II)-phen (4 mol%; 40 μM) loading with respect to LmrR, LmrR_V15pAF or variants (5 mol%; 50 μM), 1 mM **1a**, 10 mM **2b**, in 20 mM MOPS buffer (pH 7.0), 150 mM NaCl, at 4 °C for 48 h, unless noted otherwise. Yield and e.e. values are the average of at least two independent experiments, both carried out in duplicate. All error values are given as standard deviation. [a]Yields are determined by HPLC. [b]e.e. and d.r. values are determined by chiral HPLC. [c]LmrR (5 mol%; 50 μM), aniline (5 mol%; 50 μM) and Cu(II)-phen (5 mol%; 50 μM). [d]Conditions: 1.2 equiv Cu(II)-phen (6 mol%; 60 μM) loading with respect to LmrR_V15pAF or variants (5 mol%; 50 μM), 1 mM **1a**, 10 mM **2b**, in 20 mM MES buffer (pH 6.0), 150 mM NaCl, at 4 °C for 48 h. [d] Reaction with 2.5 mol% protein and 3 mol% Cu(II)-phen loading. [e] Reaction with 1 mol% protein and 1.2 mol% Cu(II)-phen loading. [f]Reaction with 0.5 mol% protein and 0.6 mol% Cu(II)-phen loading. Close-up of the hydrophobic pore in LmrR_pAF crystal structure (PDB: 6I8N). Catalytic aniline side chains (pink), Trp96 (yellow) and Met8 (red) are shown as sticks.

Mutagenesis of LmrR. A limited mutagenesis study was performed. Removal of the Cu(II)-phen binding site by mutation of the central tryptophan residues to alanine, that is, LmrR_V15pAF_W96A with Cu(II)-phen resulted in a lower yield and e.e., as expected, confirming the importance of the precise positioning of the Cu(II)-bound enolate with respect to the activated enal (Table 2, entry 9). Mutagenesis of the methionine 8, which is directly adjacent to the central

tryptophans, has been found to be beneficial for catalysis in several instances.[33] Several LmrR mutants, containing a variety of hydrophobic and charged side chains at position M8 were prepared and evaluated in catalysis (Table 2, entry 10-13). The variant LmrR_V15pAF_M8L displayed both improved stereoselectivity and reactivity compared to LmrR_V15pAF with >99/93% e.e., 6 : 1 d.r. and 82% yield (Table 2, entry 14). The catalyst loading could be lowered to 0.5 mol% without affecting the enantioselectivity (Table 2, entries 6-8, 16-17), confirming that the racemic background reaction is non-existent.

Table 3. Substrate scope of Michael addition reactions catalyzed by LmrR-based artificial enzymes



1 **2** **3a-h**

a: R^1=Ph, R^2=H
b: R^1=Ph, R^2=CH_3
c: R^1=Ph, R^2=*n*Pr
d: R^1=Ph, R^2=Ph
e: R^1=*p*-MeOPh, R^2=CH_3
f: R^1=*p*-MeOPh, R^2=H
g: R^1=*p*-Cl, R^2=CH_3
h: R^1=3-thienyl, R^2=CH_3

Entry	Product	*LmrR_V15pAF + Cu(II)-phen*			*LmrR_V15pAF_M8L + Cu(II)-phen*		
		Yield (%)[a]	**d.r.[b]**	**e.e. (%)[b]**	**Yield (%)[a]**	**d.r.[b]**	**e.e. (%)[b]**
1	**3a**	42 ± 3	/	85 ± 2	35 ± 2	/	85 ± 1
2	**3b**	65 ± 1	4 : 1	98 ± 0/86 ± 1	82 ± 1	6 : 1	>99 ± 0/93 ± 1
3	**3c**	32 ± 3	4 : 1	98 ± 0/82 ± 1	48 ± 2	5 : 1	97 ± 0/85 ± 1
4	**3d**	56 ± 6	2 : 1	61 ± 5/18 ± 2	52 ± 4	2 : 1	72 ± 3/12 ± 2
5	**3e**	72 ± 3	8 : 1	99 ± 0/85 ± 1	90 ± 2	9 : 1	>99 ± 0/85 ± 1
6	**3f**	53 ± 2	/	96 ± 1	55 ± 3	/	97 ± 0
7	**3g**	80 ± 2	7 : 1	97 ± 0/67 ± 1	88 ± 1	8 : 1	98 ± 0/80 ± 1
8	**3h**	46 ± 3	5 : 1	98 ± 0/72 ± 2	82 ± 2	6 : 1	>99 ± 0/81 ± 1

Typical conditions: 1.2 equiv Cu(II)-phen (6 mol%; 60 μM) loading with respect to LmrR_V15pAF and LmrR_V15pAF_M8L (5 mol%; 50 μM), 1 mM **1**, 10 mM **2**, in 20 mM MES buffer (pH 6.0), 150 mM NaCl, at 4 °C for 48 h, unless noted otherwise. Yield and e.e. values are the average of at least two independent experiments, both carried out in duplicate. All error values are given as standard deviation. [a]Yields are determined by HPLC. [b]e.e. and d.r. values are determined by chiral HPLC.

Investigation of substrate scope. The substrate scope of LmrR_V15pAF and LmrR_V15pAF_M8L with Cu(II)-phen was evaluated by variation of the α,β-unsaturated aldehyde and of the 2-acyl imidazole. The artificial enzyme tolerates a variety of α,β-unsaturated aldehydes. Replacing the methyl group at the β position of the unsaturated aldehyde with a propyl (**2c**) resulted in a similar e.e., whereas a phenyl at this position (**2d**) resulted in a lower e.e. (Table 3, entry 3–4). The reaction with the para-methoxy substitute 2-acyl imidazole and acrolein or crotonaldehyde both gave excellent e.e. and yield with both variants (Table 3, entry 5–6). The para-chloroe and 3-thiophene substituted 2-acyl imidazole also shows excellent results in this reaction (Table 3, entries 7-8). The mutant LmrR_V15pAF_M8L gave rise to either similar (with **3a** and **3d**) or higher yields and enantioselectivities than LmrR_V15pAF (in case of **3b**, **3c**, **3e**, **3f**, **3g** and **3h**).

Mode of action of the artificial enzyme. The combined results of the binding studies and catalysis demonstrate unequivocally that the reaction takes place thanks to a synergistic combination of iminium ion activation of the unsaturated aldehyde by the pAF residue and the Cu(II)-phenanthroline induced enolization of the ketone. The formation of an iminium ion intermediate from reaction of crotonaldehyde with the aniline moiety of LmrR_V15pAF is supported by the trapping of this intermediate with $NaCNBH_3$ to give the corresponding reductive product, as shown by mass spectrometry.(Supplementary Figure 7). The binding of the Cu(II)-bound enolate between central tryptophan residues W96/W96′ is of key importance both for the activity and selectivity of the reaction. It brings the activated nucleophile and electrophile together, resulting in efficient C-C bond formation. Moreover, it directs the attack of the nucleophile to one preferred prochiral face of the enone, resulting in excellent dia- and enantioselectivities of the product. Finally, the spatial separation of amine and Cu(II) catalytic sites ensures that the reaction can occur even though

aniline, and other amine catalysts, and Cu(II) complexes are incompatible when combined, without the protein.

Conclusions.

We have presented a design of an artificial enzyme, created by synergistic combination of an genetically encoded unnatural catalytic pAF residue with a catalytically active Cu(II) complex introduced by supramolecular binding in the hydrophobic cavity of the dimer interface of LmrR. The two key elements of the design are the promiscuity of the hydrophobic cavity of LmrR and the physical separation and judicious positioning of the two incorporated abiological catalytic sites, which circumvents problems related to incompatibility of the individual catalytic components, while allowing for efficient and selective approach of the nucleophile to the activated electrophile. The power of this approach was illustrated in the asymmetric Michael addition reaction in water, giving excellent stereoselectivity and reactivity. Finally, this study shows how the synergistic combination of two abiological catalytic groups can be employed to perform catalysis that is outside the realm of artificial enzymes containing a single abiological catalytic site. Our artificial enzyme design is highly flexible and allows for incorporation of other organocatalytic unnatural amino acids as well that it can binding other metal complexes.[33] Thus, this study provides an attractive way forward to achieving enzymatic catalysis of new-to-nature reactions.

Methods

General

Unless otherwise noted, chemicals were purchased from *Sigma Aldrich* and used without further purification. The unnatural amino acid pAzF was purchased as racemic mixture from *Bachem* (Switzerland). Plasmid pEVOL-pAzF was obtained from *Addgene* (pEVOL-pAzF was a gift from Prof. P. G. Schultz (The Scripps Research Institute), Addgene plasmid #31186).[34] *E. coli* strains NEB5-alpha and BL21(DE3) (*New England Biolabs*, USA) were used for cloning and expression. Primers were synthesized by *Eurofins MWG Operon* (Germany) and restriction endonucleases purchased from *New England Biolabs* (USA). Plasmid Purification Kits were obtained from *QIAGEN* (Germany) and DNA sequencing carried out by *GATC-Biotech* (Germany). *Pfu* Turbo polymerase was purchased from *Agilent* (USA) and Strep-tactin columns (Strep-Tactin® Superflow® high capacity) from *IBA-Lifesciences* (Germany). Concentrations of DNA and protein solutions were determined based on the absorption at 260 nm or 280 nm on a Thermo Scientific Nanodrop 2000 UV-Vis spectrophotometer. UPLC/MS analysis was performed on a Waters Acquity Ultra Performance LC with Acquity TQD detector. Separation of proteins was achieved with an Acquity UPLC BEH C8 1.7 µm 2.1x150 mm column and a linear gradient of 90% to 50% water (0.1% FA) in ACN (0.1% FA) in 15 minutes. Theoretical molecular weights of proteins were calculated using the Expasy ProtParam tool (http://web.expasy.org./protparam/). Analytical size-exclusion chromatography was carried out using a Superdex-75 10/300 GL size-exclusion column (GE Healtcare). Protein samples (100 µl) were injected and separated at a flow rate of 0.5 ml/min with 50 mM NaH_2PO_4, 150 mM NaCl buffer (pH 7.4). The column was calibrated using the standard Gel Filtration LMW Calibration Kit (GE Healtcare).

Site-directed mutagenesis

The LmrR variant used as template for all mutants in this study is LmrR, which contains mutations at 2 lysines (K55D and K59Q) that abrogate the natural DNA-binding ability of the protein.[4] Site-directed mutagenesis was performed for the preparation of all LmrR variants used in this study (QuickChange, Agilent Technologies). Starting from the previously reported plasmid, pET17b_LmrR_LM,[35] 2 primers harboring appropriate mutations were used to generate UAG-containing LmrR variants (a list of primers can be found in the Supplementary Information). The following PCR protocol was used: (1) initial denaturation at 95 ^{0}C for 1 min, (2) 16 cycles of denaturation at 98 ^{0}C for 30 s, annealing at 52-55 ^{0}C for 30 s (depending on the T_m of the primers) and extension at 72 ^{0}C for 4 min 30 s; (3) a final extension at 72 ^{0}C for 10 min. The resulting PCR product was digested with DpnI for 2 hours at 37 ^{0}C and transformed into chemically competent *E. coli* NEB5-alpha cells. A single colony was picked from LB plates containing ampicillin (100 μg/mL) and used to inoculate 5 mL of LB medium containing the same concentration of ampicillin. Bacteria were grown overnight, plasmids isolated and variants harboring the correct mutations identified by sequencing. For protein expression, the plasmids pEVOL-pAzF and pET17b_LmrR_X were co-transformed into E. coli BL21(DE3) and a single colony was used to inoculate an overnight culture for protein expression and purification.

Protein expression and purification

LmrR_pAF variants were produced and purified purified as described previously.[4] The identity of proteins and the successful reduction of pAzF were determined by mass spectrometry and the purity of the protein confirmed by SDS PAGE. Protein concentration was determined by correcting the calculated extinction coefficients for LmrR variants for the absorbance of pAF (ε_{280} = 1333 M^{-1} cm^{-1}). Analytical size-exclusion chromatography gave an elution volume (11.6 ± 0.2 ml) for all

LmrR mutants corresponded to a molecular weight of around 30 kDa, which is consistent with a homodimeric structure.

General procedure for catalytic reactions and product characterization

The catalytic solution was prepared by combining Cu(1,10-phenanthroline)$(NO_3)_2$ (Cu(II)-phen) (60 μM, 6 % catalyst loading) with LmrR_V15pAF_ or LmrR_V15pAF variants (50 μM, 5 % catalyst loading) in a final volume of 276 μL MES buffer (20 mM MES, 250 mM NaCl, pH 6.0) and incubating at 4 ^{0}C for one hour. To this mixture, 12 μL of a fresh stock solution of substrate **1** in DMF/MES buffer (50:50, 25 mM, final concentration in reaction mixture 1 mM) and 12 μL of a fresh stock solution of substrate **2** in DMF/MES buffer (50:50, 250 mM, final concentration in reaction mixture 10 mM) were added. The reaction was mixed for 2 days by continuous inversion at 4 ^{0}C. Then the product was extracted with 2 x 1 mL of ethyl acetate, the organic layers were dried on Na_2SO_4, and the solvent was evaporated under reduced pressure. The product was redissolved in 120 μl of a heptane:propan-2-ol mixture (9:1) and the yield and enantiomeric excess were determined with normal phase HPLC, using a calibration curve.

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Author Contributions

GR conceived and directed the project. ZZ performed the experimental work and analyzed the data. The authors discussed the results and wrote the manuscript together. Correspondence to GR.

Data Availability

All data that support the findings of this study are available within Figures and in the Supplementary Information or from the corresponding author upon reasonable request.

Competing interests

The authors declare no competing interests.

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